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Selective knockdown of AT₁ receptors by RNA interference inhibits Val⁵-ANG II endocytosis and NHE-3 expression in immortalized rabbit proximal tubule cells

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Abstract

Receptor-mediated endocytosis of extracellular ANG II has been suggested to play an important role in the regulation of proximal tubule cell (PTC) function. Using immortalized rabbit PTCs as an in vitro cell culture model, we tested the hypothesis that extracellular ANG II is taken up by PTCs through angiotensin type 1 receptor (AT₁; or AT_{1a}) receptor-mediated endocytosis and that inhibition of ANG II endocytosis using a selective AT₁ receptor small-interfering RNA (siRNA; AT₁R siRNA) or endocytotic inhibitors exerts a physiological effect on total and apical sodium and hydrogen exchanger isoform 3 (NHE-3) protein abundance. Western blots and live cell imaging with FITC-labeled ANG II confirmed that transfection of PTCs with a human specific AT₁R siRNA for 48 h selectively knocked down AT₁ receptor protein by $76 \pm 5\%$ ($P < 0.01$), whereas transfection with a scrambled siRNA had little effect. In nontransfected PTCs, exposure to extracellular ANG II (1 nM) for 60 min at 37°C increased intracellular ANG II accumulation by 67% (control: 566 ± 55 vs. ANG II: 943 ± 160 pg/mg protein, $P < 0.05$) and induced mitogen-activated protein kinase extracellular signal-regulated kinase (ERK) 1/2 phosphorylation ($163 \pm 15\%$ of control, $P < 0.01$). AT₁R siRNA reduced ANG II endocytosis to a level similar to losartan, which blocks cell surface AT₁ receptors (557 ± 37 pg/mg protein, $P < 0.05$ vs. ANG II), or to colchicine, which disrupts cytoskeleton microtubules (613 ± 12 pg/mg protein, $P < 0.05$ vs. ANG II). AT₁R siRNA, losartan, and colchicine all attenuated ANG II-induced ERK1/2 activation and total cell lysate and apical membrane NHE-3 abundance. The scrambled siRNA had no effect on ANG II endocytosis, ERK1/2 activation, or NHE-3 expression. These results suggest that AT₁ receptor-mediated endocytosis of extracellular ANG II may regulate proximal tubule sodium transport by increasing total and apical NHE-3 proteins.

Keywords

extracellular signal-regulated kinase 1/2; kidney; sodium transport; receptor internalization; ribonucleic acid interference

ANG II is well recognized as one of the most powerful vasoactive peptides in the regulation of body salt and fluid balance and blood pressure homeostasis in health as well as in disease (23,28,37,55). ANG II is produced and exerts its biological and physiological effects in three different manners. First, it is formed in the circulation via actions of kidney-derived renin and vascular angiotensin-converting enzyme and acts as an endocrine hormone to regulate blood pressure (7,20). Second, ANG II may be synthesized inside the cells of many target tissues,

including the kidney, heart, and brain, and, upon release, it alters the function of adjacent cells in a paracrine manner (7,12,16,29). Third, it is increasingly realized that ANG II can enter the cells via receptor-mediated endocytosis or is synthesized intracellularly, where it may act as an intracrine peptide (18,34,40,49,53,56). Although the endocrine and paracrine effects of ANG II have been studied extensively in different target tissues, the roles of intracellular ANG II remain poorly understood. The proximal tubule of the kidney is an exemplary target of endocrine, paracrine, and intracellular actions of the octapeptide (23,37,44,45,56). Because proximal tubules physiologically reabsorb > 65–70% of the filtered sodium chloride and sodium bicarbonate loads, increased re-absorption of these solutes and fluid by ANG II may contribute to sodium and fluid retention and consequently the development of hypertension (23,28,37,55).

Previous studies have shown that luminal or peritubular capillary administration of ANG II results in biphasic responses, with picomolar concentrations stimulating proximal sodium and fluid transport but nanomolar concentrations inhibiting transport (13,23,37). Conversely, systemic administration of angiotensin type 1 (AT₁) receptor antagonists has an inhibitory effect on ANG II-induced responses (28,32,55,57). These studies suggest that extracellular ANG II acts on cell surface receptors to elicit endocrine and/or paracrine responses. However, sustained exposure of AT₁ receptors to ANG II also triggers receptor-mediated endocytosis of the receptor-agonist complex to the cells (2,18,22,25,34,49,55,57). Although endocytosis plays an important role in receptor desensitization and subsequent resensitization after internalized receptors recycle back to the membrane (18,25), increasing evidence suggests that internalized ANG II may serve as an intracellular peptide and play an intracrine role (12,34,40,44,45,54, 56). We recently demonstrated that extracellular ANG II accumulates in renal cortical endosomes and intermicrovillar clefts after long-term ANG II infusion (55) and in cultured rabbit PTCs via AT₁ receptor-mediated and cytoskeleton microtubule- and tyrosine phosphatase-dependent mechanisms (34). We further showed that pharmacological blockade of endocytosis affected intracellular cAMP production (34) and activation of nuclear factor (NF)- κ B (54), whereas microinjection of ANG II directly in single PTCs induced calcium mobilization (56). Yet it remains unclear how the intracellular signaling induced by ANG II is translated into the effects associated with proximal tubule sodium transport.

In the present study, we used immortalized rabbit proximal tubule cells (PTCs) as an in vitro cell model to test the hypothesis that extracellular ANG II is taken up by PTCs through AT₁ (or AT_{1a}) receptor-mediated endocytosis and that internalized ANG II acts as an intracellular peptide to regulate expression of the sodium and hydrogen exchanger isoform 3 (NHE-3). We chose NHE-3 as an indicator of proximal tubule sodium transport because most transcellular sodium chloride and sodium bicarbonate absorption is mediated by NHE-3, which is expressed almost exclusively in proximal tubule apical membranes in the kidney (1,31,36,51).

MATERIALS AND METHODS

Cells

Immortalized rabbit PTCs (vEPT) were obtained from the American Type Culture Collection (ATCC), and their morphological and biochemical characteristics have already been described (13,27,41). The vEPT cell line was established and deposited in ATCC by Hopfer et al. (27) and Romero et al. (41). These cells were directly derived from primary cultures of the S₁ segment of proximal tubules and microdissected specifically from the superficial renal cortex of a normal male New Zealand rabbit. The cells were immortalized using a recombinant retrovirus encoding SV40 large-T antigen (27,41). After transformation, the vEPT cells retain morphological and biochemical properties of rabbit PTC, growing to confluent monolayers with cuboidal to columnar shapes, some apical microvilli, tight junctions, and convoluted basolateral membranes (27). The vEPT cells also retain electrolyte transport activities (glucose

cotransporters, sodium-potassium-ATPase, sodium/hydrogen exchangers) and expression of receptors and signaling transduction pathways for ANG II (13,27,41). Unlike cells of the rodent kidney that express both AT_{1a} and AT_{1b} receptors (11,39,52), these PTCs predominantly express the AT₁ receptor, which shares a similar genomic homology with human AT₁ or rodent AT_{1a} receptors (11). However, immortalized PTCs appear to have lower conductances and fewer microvilli than PTCs in vivo (27). Human embryonic kidney cells (HEK 293) and HEK 293 cells stably expressing AT_{1a} receptors were a gift from Dr. Walter G. Thomas of Baker Heart Research Institute, Melbourne, Australia (46,47).

Chemicals

DMEM nutrient mixture and Ham's F-12 (DMEM-F-12), trypsin, heat-inactivated FBS, and antibiotics (penicillin and streptomycin) were purchased from ATCC. Human Val⁵-ANG II and ANG II enzyme immunoassay kits were obtained from Biochem (Peninsula). The AT₁ receptor antagonist losartan was a gift from Merck Pharmaceuticals; and the AT₂ receptor antagonist PD-123319 was donated by Pfizer; FITC-labeled ANG II was purchased from Invitrogen (Molecular Probes); angiotensin type 1 receptor small-interfering RNA (AT₁R siRNA) and rabbit polyclonal AT₁ receptor antibody targeting the NH₂-terminal extracellular domain of the human AT₁ receptor, scrambled siRNA and transfection reagents, the mouse monoclonal antibody targeting phosphorylated mitogen-activated protein (MAP) kinase extracellular signal-regulated kinase (ERK) 1/2, the goat polyclonal antibody targeting NHE-3, and the goat polyclonal megalin antibody and their respective secondary donkey anti-goat IgG-FITC were obtained from Santa Cruz. The rabbit polyclonal antibody targeting total MAP kinase ERK1/2 was purchased from Cell Signaling. Western blot supplies were purchased from Amersham. Colchicine was obtained from Calbiochem as described previously (17,34).

Cell culture

Unless specified elsewhere, immortalized rabbit PTCs (passages 8–12) were subcultured in six-well plates in complete DMEM-F-12 growth medium supplemented with 50 nM hydrocortisone, 5% heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (46). HEK 293 cells or HEK 293 cells stably expressing AT_{1a} receptors were grown in DMEM supplemented with 10% FBS with G418 (200 µg/ml; GIBCO) added in the medium for the latter cells to retain AT_{1a} receptor expression (46,47). All cells were maintained at 37°C and 95% O₂-5% CO₂ and fed every 2 days. After reaching 80% confluence, they were starved in serum-free medium for 24 h before the experiment, as described previously (34,41,54,56). To further verify the properties of vEPT cells as the PTC culture model, we examined whether these cells express megalin (1:50) and NHE-3 (1:100), two major proteins specifically identified in PTCs using immunofluorescence imaging (1,6,9,31,36). For negative controls of immunofluorescence of megalin and NHE-3 expression, PTCs grown on cover slips were incubated with the secondary donkey anti-goat IgG-FITC only without prior incubation with the primary megalin or NHE-3 antibodies. Furthermore, respective blocking peptides were also used to verify the specificity of these antibodies.

Knockdown of AT₁ receptor expression by AT₁R siRNA

We have recently shown that immortalized rabbit PTCs express the AT₁ receptor, which shares a similar genome with human AT₁ or mutant AT_{1a} receptors (34,54,56). We further showed that losartan blocks AT₁ receptor-mediated ANG II endocytosis and ANG II-induced activation of NF-κB in these cells (34,54). However, losartan is unable to distinguish between AT_{1a} and AT_{1b} receptors and it blocks only cell surface receptors because it is internalized poorly (10,11). In the present study, we chose a human AT₁ receptor siRNA to knock down AT₁ receptor expression, an approach similar to using an AT_{1a} receptor siRNA to knock down AT_{1a} receptor expression in nonrenal cells (8,50). We first determine the time course of the

effects of AT₁R siRNA on AT₁ receptor and NHE-3 expression. Briefly, immortalized rabbit PTCs were subcultured to 80% confluence in six-well plates before transfection ($n = 6$ each group, repeated two times). The first group was treated with serum-free medium for 24 h as a control. Three groups were transfected with the same concentration of a human AT₁ receptor-specific 20–25 nucleotide siRNA (AT₁R siRNA) according to the manufacturer's instructions (Santa Cruz). The cells were harvested 24, 48, or 72 h after transfection, respectively, to determine AT₁ receptor and NHE-3 proteins using Western blot as described (33–35,54,56). We next determined the specificity of the effects induced by AT₁R siRNA to ensure that it specifically knocks down AT₁ receptors. Three different approaches were used to verify the AT₁R siRNA's specificity of RNA interference. First, an additional group of cells was transfected with a negative, non-AT₁ receptor-targeting, scrambled siRNA as we described previously (34,54). Second, the effects of AT₁R siRNA on AT₁ receptor expression were evaluated using live cell fluorescence imaging of FITC-labeled ANG II (Molecular Probes) as described (34,56). Third, we determined whether the AT₁R siRNA we used could specifically knock down AT_{1a} receptor expression in HEK 293 cells with stable expression of AT_{1a} receptors (46,47). After transfection, the cells were allowed to grow for 48 h to ensure > 70–80% knock down of AT₁ receptor proteins before endocytosis was studied. For negative controls of AT₁ receptor fluorescence imaging in PTCs, cells grown on cover slips were first incubated with losartan (10 μ M) for 30 min before imaging with FITC-labeled ANG II.

Effects of AT₁ receptor knockdown on receptor-mediated endocytosis of extracellular Val⁵-ANG II

To determine whether knocking down the AT₁ receptor blocks receptor-mediated endocytosis of extracellular ANG II in PTCs, the growth medium was first removed, and cells were washed with warm serum-free medium after transfection. Five groups of transfected or nontransfected PTCs ($n = 6$ wells each) were then treated as follows: 1) nontransfected PTCs with serum-free medium as a control; 2) nontransfected PTCs with ANG II (1 nM) for 60 min at 37°C; 3) AT₁R siRNA-transfected PTCs with ANG II (1 nM) for 60 min at 37°C; 4) scrambled-transfected PTCs with ANG II (1 nM) as a negative control; and 5) non-AT₁R siRNA-transfected PTCs with ANG II (1 nM) and losartan (10 μ M) compared with ANG II plus AT₁R siRNA. Experiments were also performed using nontransfected PTCs that were treated with cold, colchicine, or phenylarsine oxide (PAO) for comparison with AT₁R siRNA as described (34). After incubation, the medium was removed, and cells were washed with ice-cold acid buffer to remove membrane-bound agonist before extraction of protein samples for ELISA of ANG II as described previously (34,54–56).

Effects of blockade of ANG II endocytosis by AT₁R siRNA, losartan, and other endocytotic inhibitors on MAP kinase ERK1/2 activation and NHE-3 expression

To determine whether AT₁ receptor-mediated endocytosis of extracellular ANG II is involved in regulating proximal tubule sodium transport, total and phosphorylated ERK1/2 (33–35,54,56) and lysate and cell-surface NHE-3 proteins were evaluated by Western blot as described (1,5,31,32). Nontransfected and AT₁R siRNA-transfected PTCs were subcultured to 60–80% confluence in six-well plates before being treated with vehicle (serum-free medium), ANG II (1 nM), ANG II plus losartan (10 μ M), ANG II plus the cytoskeleton microtubule inhibitor colchicine (1 μ M; see Refs. 17,34, and 43), or ANG II plus the tyrosine phosphatase inhibitor PAO (1 μ M) for 60 min at 37°C (22,34,44). In separate experiments, PTCs were incubated with ANG II for 60 min at 4°C to inhibit AT₁ receptor endocytosis for comparisons. After treatment, the cells were washed, and protein samples were extracted for measurement of total and phosphorylated ERK1/2 as described (33–35) as well as total and surface NHE-3 proteins by Western blot (1,5,31,32). To measure cell-surface NHE-3 protein, standard cell-surface biotinylation procedures were followed to obtain cell-surface proteins for Western blot as described previously (1,5,32). Briefly, subconfluent PTCs were transfected with AT₁R siRNA

or scrambled RNA or treated with losartan or colchicine, as described above. After treatment, PTCs were washed two times with ice-cold PBS, and cell surface proteins were biotinylated by incubating the cells with buffer containing 1.5 mg/ml sulfo-NHS-SS-biotin, 10 mM triethanolamine (pH 7.4), 2 mM CaCl_2 and 150 mM NaCl for ~2 h at 4°C (5). The cells were washed with quenching buffer (PBS containing 1 mM MgCl_2 , 0.1 mM CaCl_2 , and 100 mM glycine) for 20 min to clear unbound biotin. PTCs were lysed with a modified RIPA buffer (50 mM Tris · HCl, 50 mM 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM Na_3VO_4 , and 1 mM NaF, pH 7.4) and centrifuged, and lysates were collected. The samples were incubated with avidin-agarose beads (Pierce) overnight (or 16 h) at 4°C. Finally, avidin-agarose beads were washed sequentially with 50 mM Tris buffer (pH 7.4) containing 100 mM NaCl and 5 mM EDTA (1st wash), 50 mM Tris buffer containing 100 mM NaCl (pH 7.4) (2nd wash), or 50 mM Tris buffer only (pH 7.4; 3rd wash; see Ref. 5). Biotinylated proteins were recovered by heating the samples at 95°C with $\times 2.5$ loading buffer before Western blot was performed (see below and Ref. 1,5, and 32).

Western blots of AT₁ receptor proteins, NHE-3, β -actin, and total and phosphorylated ERK1/2

For Western blots of protein abundance, PTCs were washed two times with ice-cold PBS after treatment and lysed with a modified RIPA buffer as described above. Protein samples were extracted, and concentrations were determined using a bicinchoninic acid protein assay kit (Pierce) and Prism 4.0. PTC protein samples (10 µg each) were electrophoretically separated on 8–16% Tris-glycine gels at 120 volts for 1.5–2 h. After SDS separation, proteins were transferred to Millipore Immobilon-P membranes using a Bio-Rad Trans-Blot Semi-Dry system powered by a Bio-Rad Power-Pac HC (25 V, 0.12 A, 1.5 h). The membranes were blotted overnight at 4°C with 5% nonfat dry milk and incubated for 3 h at room temperature with a rabbit polyclonal antibody against the human AT₁ receptor (1:200; Santa Cruz; see Refs. 34,54, and 56). The specificity of this antibody has been verified previously (34,54,56). Total and cell-surface NHE-3 proteins were detected using a goat polyclonal NHE-3 antibody targeting the COOH-terminus of human origin of NHE-3 (Santa Cruz). Specificity of the NHE-3 antibody was verified using a selective NHE-3 antigen blocking peptide, SC-10163P (Santa Cruz). Total and phosphorylated ERK1/2 were determined as we described previously (33–35). To ensure equal protein loading, the same membranes were treated with stripping buffer (Pierce) for 20 min, blotted with 5% nonfat dry milk, and reprobed with a mouse anti- β -actin monoclonal antibody at 1:2,000 (Sigma-Aldrich). Western blot signals were detected using enhanced chemiluminescence (Amersham) and analyzed using a microcomputer imaging device with a digital camera (MCID; Imaging Research).

Statistical analysis

Results are expressed as means $\pm$ SE. For measurement of intracellular ANG II levels, 6–12 samples from two separate experiments were collected for each treatment and assayed in duplicate. For Western blot data, three samples from two separate experiments were performed, with each treatment assayed in duplicate. Comparisons between two treatments were made by Student's unpaired *t*-test. Comparisons between more than two treatments were made by one-way ANOVA, followed by a Newman-Keul's test for multiple comparisons. *P* < 0.05 was considered significant.

RESULTS

Properties of immortalized rabbit PTC

The morphological and electrolyte transport properties of immortalized rabbit PTCs have been reported previously (13,27,41). As shown in Fig. 1, the vEPT cells grew to monolayers of cuboidal to columnar shapes (Fig. 1, A and B), and immunofluorescence staining using specific

megalin or NHE-3 antibodies shows that these cells expressed abundant megalin (Fig. 1C) and NHE-3 proteins (Fig. 1D). Negative controls of megalin or NHE-3 immunofluorescence are shown in Fig. 1, E and F, respectively. Megalin and NHE-3 are two specific proteins expressed in PTC of the kidney. Megalin is the major endocytic receptor responsible for uptake of glomerular filtrated proteins, metals, and nutrients in the S₁ segment of proximal tubules (6, 9), whereas NHE-3 is the major transport protein responsible for transcellular sodium and bicarbonate reabsorption in proximal tubules (36,51).

Time-dependent knockdown of AT₁ receptor expression by AT₁R siRNA

The rationale for using AT₁R siRNA as a tool was to knock down AT₁ receptor expression both within and on the cell membrane. Figure 2A shows the time course of the inhibitory effects of AT₁R siRNA on AT₁ receptor and NHE-3 protein abundance in immortalized rabbit PTCs. Compared with control (100%), AT₁R siRNA caused time-dependent decreases in AT₁ receptor proteins in immortalized rabbit PTCs, which was evident at 24 h ($48 \pm 5\%$, $P < 0.01$ vs. control) and peaked at 48 h after transfection ($18 \pm 3\%$, $P < 0.01$ vs. control or 24 h). The effect persisted at 72 h after transfection ($36 \pm 6\%$, $P < 0.01$ vs. control; Fig. 2). Interestingly, AT₁R siRNA did not significantly inhibit NHE-3 expression at 24 h but markedly decreased NHE-3 expression at 48 h after transfection ($42 \pm 3\%$ of control, $P < 0.01$). NHE-3 expression returned to control at 72 h after transfection.

Specificity of the effects of AT₁R siRNA on inhibition of AT₁ receptor expression

The specificity of AT₁ receptor knockdown by AT₁R siRNA was verified using a combination of live cell FITC-ANG II imaging, Western blot of AT₁ receptor protein, and HEK 293 cells stably expressing AT_{1a} receptors. Figure 2B shows that AT₁R siRNA specifically knocked down AT_{1a} receptor expression by $79 \pm 3\%$ in HEK 293 cells stably expressing AT_{1a} receptors ($P < 0.01$). Nontransfected HEK 293 cells expressed little AT_{1a} receptors. Figure 3 shows that FITC-ANG II fluorescence staining was markedly reduced in AT₁R siRNA-transfected immortalized rabbit PTCs (Fig. 3C) compared with nontransfected cells (Fig. 3A). The specificity of FITC-ANG II fluorescence was further verified using negative controls in which pretreatment of PTCs with losartan for 30 min blocked FITC-ANG II labeling (Fig. 3B). Western blots of AT₁ receptor protein and semiquantitated changes in response to AT₁R-siRNA or a scrambled siRNA are shown in Fig. 3, D and E, respectively. Compared with control, ANG II increased AT₁ receptor protein expression by $30 \pm 3\%$, as expected (34), whereas AT₁R siRNA decreased AT₁ protein abundance by $76 \pm 5\%$ ($P < 0.01$ vs. control). By contrast, a scrambled, non-AT₁ receptor-selective RNA had little effect on AT₁ receptor protein expression. Taken together, these results suggest that the AT₁R siRNA we used is a potent and selective inhibitor of AT₁ receptor expression in both immortalized rabbit PTCs and HEK 293 cells stably expressing AT_{1a} receptors.

Effects of AT₁R siRNA and losartan on AT₁ receptor-mediated endocytosis of extracellular ANG II

We have recently shown that losartan or candesartan blocked AT₁ receptor-mediated accumulation of extracellular ANG II in immortalized rabbit PTCs or renal cortical endosomes of ANG II-infused rats (34,55). In the present study, incubation of PTCs with 1 nM extracellular ANG II for 60 min at 37°C increased ANG II levels by 67%, from 566 ± 55 to 943 ± 160 pg/mg protein ($P < 0.05$; Fig. 4). Transfection of AT₁R siRNA in ANG II-treated cells reduced ANG II to a level not significantly different from control [449 ± 55 pg/mg protein, not significant (NS)]. By contrast, the negative, non-AT₁ receptor-targeting scrambled siRNA had no effect on ANG II endocytosis (724 ± 41 pg/mg protein, NS; Fig. 4). To further support the effect of AT₁R siRNA on AT₁ receptor-mediated ANG II endocytosis, losartan (10 μM) was added to a separate group of PTCs to block cell-surface AT₁ receptors before exposure to ANG

II. Losartan was found to inhibit ANG II-induced endocytosis to a similar extent in PTCs (Fig. 4; see Ref. 34). Likewise, cold, colchicine, or PAO all blocked AT₁ receptor-mediated endocytosis of ANG II as expected (34; Fig. 5).

Effects of selective knockdown of AT₁ receptors by AT₁R siRNA on NHE-3 expression

ANG II has profound effects on proximal tubular sodium transport by regulating apical membrane sodium and hydrogen antiporter NHE-3 activity (36,38,42,51). Although transfection of AT_{1a} receptor-expressing CHO cells with a siRNA targeting AT_{1a} receptors has been found to inhibit ANG II-stimulated ⁴⁵Ca²⁺ concentration uptake (50) and lower blood pressure (8), it is not known whether an AT₁R siRNA could block ANG II-stimulated NHE-3 expression in immortalized rabbit PTCs, an index of proximal tubular sodium transport. Figure 6 shows that ANG II increased NHE-3 protein expression by $35 \pm 3\%$ ($P < 0.05$), which was inhibited by pretransfection of PTCs with AT₁R siRNA to a level significantly below control. By contrast, ANG II-stimulated NHE-3 expression was not significantly altered by scrambled RNA, indicating that the effect of AT₁R siRNA on ANG II-stimulated NHE-3 expression was specific for AT₁ receptors.

Effects of cold condition and losartan on NHE-3 expression

In a previous study, we showed that both cold condition and losartan significantly inhibited AT₁ receptor-mediated endocytosis of extracellular ANG II in immortalized rabbit PTCs (34). We tested whether the cold condition and losartan have any functional effects on AT₁ receptor-mediated ANG II accumulation in these cells. As expected, ANG II increased NHE-3 protein levels by $30 \pm 5\%$ over control (Fig. 5). Incubation of PTCs at 4°C or pretreatment of PTCs with losartan (10 μM) for 30 min at 37°C before exposure to ANG II (1 nM) effectively blocked ANG II-stimulated NHE-3 expression (Fig. 7).

Effects of endocytotic inhibitors on ANG II-stimulated NHE-3 expression

Cytoskeleton microtubules and tyrosine phosphatases have been shown to play an important role in AT₁ receptor-mediated endocytosis of ANG II and in ANG II-stimulated sodium transport in PTCs (2,17,22,43,44). We tested whether endocytotic inhibitors block ANG II-stimulated NHE-3 expression by suppressing AT₁ receptor-mediated endocytosis. Figure 8 shows that colchicine, a selective inhibitor of cytoskeleton microtubules, and PAO, a selective inhibitor of tyrosine phosphatases, completely blocked ANG II-induced NHE-3 expression. These results suggest that AT₁ receptor-mediated endocytosis of extracellular ANG II is facilitated by the actions of cytoskeleton microtubules and/or tyrosine phosphatases and that, after endocytosis, ANG II may exert an important role in stimulating NHE-3 expression in PTCs.

Effect of blockade of AT₁ receptor-mediated endocytosis of ANG II by AT₁R siRNA, losartan, and colchicine on cell-surface NHE-3 abundance

To determine whether blockade of ANG II endocytosis by AT₁R siRNA, losartan, or colchicine affects cell-surface NHE-3 proteins, an index of NHE-3 insertion in the cell membrane, we measured cell-surface NHE-3 protein after biotinylation (1,5,14,26,32). Figure 9 shows that cell-surface NHE-3 proteins were also significantly reduced by AT₁R siRNA, losartan, or colchicine, suggesting that blocking endocytosis of extracellular ANG II not only reduced NHE-3 protein synthesis but also decreased NHE-3 insertion in the cell membrane.

Effects of AT₁R siRNA, losartan, and colchicine on ANG II-induced MAP kinase ERK1/2 activation

ANG II is known to activate MAP kinases and induce ERK1/2 phosphorylation in different cardiovascular and renal cells (11,21,33,47). Activation of MAP kinase ERK1/2 plays an

important role in stimulating expression and transcription of growth factors, cytokines, and transporter proteins. We tested whether AT₁R siRNA, losartan, and colchicine decrease NHE-3 protein expression in PTCs in part by inhibiting MAP kinase ERK1/2 activation. Figure 10 shows that incubation of PTCs with ANG II (1 nM) for 60 min at 37°C increased phosphorylated ERK1/2 by $60 \pm 5\%$, whereas total ERK1/2 was not affected. Pretreating PTCs with AT₁R siRNA, losartan, or colchicine before exposing them to ANG II (1 nM) all effectively blocked ANG II-induced activation of ERK1/2, suggesting that ANG II activation of ERK1/2 in PTCs may partly mediate ANG II-increased NHE-3 abundance in cell lysates and apical membranes.

DISCUSSION

We have recently shown that extracellular ANG II (Val⁵-ANG II) was internalized in immortalized rabbit PTCs via AT₁ receptor-mediated endocytosis and that blocking ANG II endocytosis by means of losartan and endocytotic inhibitors suppressed ANG II-induced decreases in basal and forskolin-stimulated cAMP accumulation (34). Because inhibition of cAMP signaling by ANG II plays an important role in ANG II-stimulated sodium and fluid transport in proximal tubules (23,26,37,45), we reasoned that, after internalization, ANG II may regulate proximal tubule transport by inhibiting cAMP production (34). However, we did not study the effects of inhibition of ANG II endocytosis on proximal tubular transport function in that study. The major objective of the current study was to further determine whether inhibition of AT₁ receptor-mediated endocytosis of extracellular ANG II by a human AT₁ receptor-specific AT₁R siRNA, losartan, or endocytotic inhibitors plays a functional role in immortalized rabbit PTCs by decreasing total and apical membrane NHE-3 protein expression. In the present study, we found that Val⁵-ANG II significantly increased the abundance of total and cell-surface NHE-3 proteins, and this effect was blocked by pretransfection of PTCs with AT₁R siRNA (equivalent to AT_{1a} receptors in rodents; Fig. 6 and Refs. 8 and 50), pretreatment with losartan (Fig. 6), which blocks cell-surface AT₁ receptors, or colchicine, a cytoskeleton microtubule inhibitor important for intracellular protein trafficking (Fig. 8 and Refs. 34,43, and 44). ANG II-induced NHE-3 protein expression appears to be associated with activation of MAP kinase ERK1/2, which were also blocked by AT₁R siRNA, losartan, and colchicine (Fig. 10). Taken together, our results provide further evidence that extracellular ANG II is taken up by PTCs via AT₁ (or AT_{1a}) receptor-mediated, cytoskeleton microtubule-dependent mechanisms, and that, after endocytosis, internalized ANG II may regulate proximal tubule sodium transport by increasing expression of NHE-3 proteins and/or promoting NHE-3 trafficking to the cell membrane.

We first evaluated the role of AT₁ (rodent AT_{1a}) receptors in receptor-mediated endocytosis of extracellular ANG II in PTCs and, in particular, whether AT_{1b} participates in this process when AT_{1a} is knocked down by an AT₁R siRNA. We and others have previously shown that AT₁ receptor blockers, such as losartan or candesartan, blocked ANG II accumulation in the whole kidney (49,55,57), the cortical endosomes of ANG II-infused rat kidney (55), or ANG II endocytosis in immortalized rabbit PTCs (34). However, rodent kidneys express two isoforms of AT₁ receptors, AT_{1a} and AT_{1b} (11). Although AT_{1a} plays a predominant role in the well-described actions of ANG II (11), it has been suggested that AT_{1b} may partially take over when AT_{1a} is absent (10,11,39,52). Unfortunately, AT₁ receptor blockers pharmacologically block both AT_{1a} and AT_{1b} and therefore are not suitable for separating the role of these two receptors. In the present study, we used a human specific AT₁R siRNA that knocks down primarily AT_{1a} because the two receptor genomes share a similar homology (34,54). Although RNA interference has higher specificity of inhibition of target proteins or signaling molecules than chemical compounds, nonspecific effects still pose a major problem for the technique. To determine the specificity of the AT₁R siRNA in knocking down AT₁ receptor protein expression, three different approaches were used in the present study. First,

FITC-labeled ANG II was used for live cell imaging of AT₁ receptor expression in PTCs with or without transfection of AT₁R siRNA (Fig. 3). We found that AT₁R siRNA markedly reduced FITC-ANG II staining in live PTCs. Second, Western blot showed that AT₁R siRNA effectively knocked down AT₁ receptor protein abundance in a time-dependent manner with a peak inhibition at 48 h after transfection (> 76%; Fig. 2). Third, the specificity of the AT₁R siRNA was further confirmed in HEK 293 cells stably expressing AT_{1a} receptors (Fig. 3). This level of efficiency in knocking down AT₁ receptor proteins by AT₁R siRNA is consistent with previous reports of Vazquez et al. (50) and Chen et al. (8). In those studies, transfection of CHO cells with a double-stranded RNA AT₁ receptor decreased AT_{1a} receptor expression by 80% (50), whereas in vivo transfection of mice with an Ad-AT_{1a} small-hairpin RNA reduced AT₁ receptors by 70% in the subfornical organ of the brain (8). AT_{1a} receptor knockdown completely blocked ANG II-stimulated calcium uptake in CHO cells (50) or decreased blood pressure when microinjected in the nucleus of the solitary tract (8). Yet there was no study that has specifically used AT₁ or AT_{1a} receptor-specific siRNAs to study receptor-mediated ANG II endocytosis in PTC.

In the present study, exposure of immortalized rabbit PTCs to extracellular ANG II increased intracellular ANG II by 67%, whereas AT₁R siRNA blocked ANG II endocytosis and reduced ANG II to the control level (Fig. 4). As a positive control, losartan also blocked ANG II endocytosis (Fig. 4). Because the scrambled siRNA did not knock down AT₁ receptor expression per se, nor did it block receptor-mediated ANG II endocytosis (Figs. 2 and 3), we can reasonably conclude that the effect of AT₁R siRNA was specific for AT₁ receptors. Although both AT₁R siRNA and losartan had similar effects, losartan exerts its effects primarily by blocking cell-surface AT₁ receptors (11,37), whereas AT₁R siRNA inhibits AT₁ receptor expression and therefore fewer receptors are available to mediate ANG II endocytosis (8,34,50,54). Therefore, comparison of the effects induced by AT₁R siRNA and losartan suggests that AT_{1b} receptors may play little if any role in overall AT₁ receptor-mediated ANG II endocytosis in PTCs. This is not entirely surprising because rabbit PTCs normally do not express AT_{1b} receptors (3,34,54), and even in the rat kidney AT_{1a} predominates while AT_{1b} accounts for only a small proportion of AT₁ receptors (11).

We next determined whether blocking AT₁ receptor-mediated endocytosis of extracellular ANG II plays a physiological role in immortalized rabbit PTCs. ANG II receptor-mediated endocytosis of ANG II has been demonstrated in vascular smooth muscle cells (2,22) and renal epithelial cells such as HEK 293 cells expressing AT_{1a} receptors (3,25) and OK cells (45). Previous studies have suggested that the primary role of receptor-mediated endocytosis is to serve as the major mechanism in desensitizing cellular responses to ANG II stimulation by moving the peptide-receptor complex rapidly in the cell after binding to cell-surface receptors and initiating intracellular signaling (18,25,46). This mechanism plays an important role in the acute physiological regulation of blood pressure and cardiovascular and renal function by ANG II. Although it is commonly accepted that the receptor does recycle back to the cell membrane, the fate and role of internalized ANG II remain poorly understood. It has been suggested that internalized ANG II may be degraded in endosomes after it is dissociated from the receptor (18,25,46); however, increasing evidence suggests that this process may play an additional role other than simply desensitizing the receptor, at least in PTCs. Schelling et al. (43,44) showed that ANG II-dependent proximal tubule sodium transport requires receptor-mediated endocytosis, because apical ANG II-stimulated ²²Na flux was inhibited by PAO, a tyrosine phosphatase inhibitor that blocks AT₁ receptor internalization. Using LLC-PK cells expressing rabbit AT₁ receptors, Becker et al. (3) reported that ANG II-stimulated phospholipase A₂ and sodium flux were also associated with AT₁ receptor-mediated endocytosis. Furthermore, in OK cells, apical AT_{1a} receptors are internalized and activate G proteins to inhibit cAMP signaling (45). Consistent with these early studies, we recently showed that endocytotic inhibitors that block receptor-mediated endocytosis inhibited ANG II accumulation in PTCs,

and these effects were associated with inhibition of both basal and forskolin-induced cAMP production (34).

In the present study, we chose NHE-3 as a functional index of proximal tubule transport function because 65–70% of glomerular filtered sodium and bicarbonate load is reabsorbed by PTCs via actions of NHE-3 (36,38,42). NHE-3 is the major isoform of sodium and hydrogen exchangers, which are expressed primarily in apical membranes of early S₁ proximal tubules (1,5,31,36). Noonan et al. (38) have shown that mice with NHE-3 knockout develop severe absorptive defects in the kidney and therefore cannot reabsorb the filtered load of sodium and fluid. ANG II has been shown to acutely stimulate NHE-3 activity and thus sodium flux or transport in PTCs or other epithelial cells via protein kinase-mediated intracellular signaling (14,30,32,42). The acute effects of ANG II-stimulated NHE-3 activity and sodium transport are thought to be because of activation of cell-surface AT₁ receptors, but whether receptor-mediated endocytosis plays any regulatory role in NHE-3 expression in PTCs has not been investigated previously to our knowledge. In the present study, we measured the abundance of total cell lysates and cell-surface NHE-3 protein levels in rabbit PTCs transfected with an AT₁R siRNA or treated with losartan or other endocytotic inhibitors (Figs. 6–9). We found that ANG II significantly increased NHE-3 protein abundance and that blockade of receptor-mediated ANG II endocytosis in PTCs by AT₁R siRNA, losartan, or colchicine was all associated with significant decreases in total (Figs. 6–8) and cell-surface (Fig. 9) NHE-3 protein abundance. These results therefore suggest that receptor-mediated endocytosis of extracellular ANG II does at least play a role in ANG II-regulated proximal tubule sodium transport through its actions on total and apical membrane NHE-3 expression or insertion.

ANG II-increased NHE-3 abundance and the effects of blockade of receptor-mediated ANG II endocytosis by AT₁R siRNA, losartan, and colchicines may involve activation of MAP kinase ERK1/2. In the present study, we found that ANG II increased phosphorylated ERK1/2 in immortalized rabbit PTCs, which was significantly attenuated by AT₁R siRNA, losartan, and colchicine (Fig. 10). We interpret these findings as suggesting that receptor-mediated ANG II endocytosis plays at least a partial role in activation of ERK1/2. ANG II has been shown to stimulate either G_q-coupled receptors to activate phospholipase C (PLC)/protein kinase C (PKC) signaling or G_s-coupled receptors to activate adenylate cyclase/protein kinase A (PKA) signaling (11,13,34,45). Both PLC/PKC and cAMP/PKA signaling can activate MAP kinase mitogen/extracellular signal-regulated kinase 1/2, leading to phosphorylation of ERK1/2 (21, 33,35,39,47). Activation of MAP kinase ERK1/2 not only phosphorylates many signaling proteins but also plays an important role in stimulating expression and transcription of growth factors, inflammatory cytokines, and transporter proteins. For example, Bianchini et al. (4) reported that activation of MAP kinase ERK1/2 plays a predominant role in activation of NHE-1 in a nonrenal epithelial cell line. In MDCK cells, an intercalated cell line, aldosterone induced rapid activation of NHE-3 through phosphorylated ERK1/2 (19). Finally, Tsuganezawa et al. (48) showed that c-SRC/ERK1/2 mediates acid-activated NHE-3 in OKP cells, a PTC line. Taken together, these findings suggest that MAP kinase ERK1/2 signaling may serve as an alternate pathway mediating internalized ANG II-increased NHE-3 protein synthesis in immortalized rabbit PTCs.

In summary, the present study demonstrates that extracellular ANG II is taken up by immortalized rabbit PTCs through AT₁ (AT_{1a}) receptor-mediated endocytosis, and after endocytosis ANG II acts as an intracellular peptide to induce expression of NHE-3, in part via activation of MAP kinase ERK1/2. This conclusion is supported by the findings that pretreatment of PTCs with an AT₁R siRNA to knock down AT₁ receptor protein expression, losartan to block cell surface AT₁ receptor-mediated endocytosis, and colchicine to disrupt cytoskeleton microtubules and intracellular protein trafficking all blocked ANG II-induced NHE-3 protein expression and/or insertion to the cell membrane. Thus our results suggest that

inhibition of ANG II endocytosis may affect expression and/or transport of NHE-3 in apical membranes of immortalized rabbit PTCs so that internalized ANG II may play a physiological role in regulating sodium transport in rabbit proximal tubules.

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Fig. 1. Morphological and biochemical properties of immortalized rabbit proximal tubule cells (PTCs). *A*: phase-contrast of low magnification of PTC monolayers ($\times 10$). *B*: phase-contrast of higher magnification of PTC monolayers showing their characteristic cuboidal and/or columnar shapes ($\times 40$). *C*: immunofluorescence micrograph showing PTC-specific megalin protein expression in green and the nuclei in blue (stained by 4,6'-diamidine-2-phenylindole; $\times 100$). *D*: immunofluorescence micrograph showing sodium and hydrogen exchanger isoform 3 (NHE-3) protein expression ($\times 40$). *E*: negative control of megalin immunofluorescence for *C*. *F*: negative control of NHE-3 immunofluorescence for *D*.

A Time-course of RNA interference by AT₁R siRNA **B** Specificity of RNA interference by AT₁R siRNA **Fig. 2.**

The time-dependent responses and specificity of the effects of RNA interference by angiotensin type 1 receptor small-interfering RNA (AT₁R siRNA) on AT₁ receptor and/or NHE-3 protein expression in immortalized rabbit PTCs or HEK 293 cells stably expressing AT_{1a} receptors, as determined by Western blot. *A*: the time course of RNA interference by AT₁R siRNA in rabbit PTCs at 24, 48, and 72 h after transfection. *B*: the specificity of RNA interference by AT₁R siRNA in HEK 293 cells stably expressing AT_{1a} receptors. In rabbit PTCs, the effects of AT₁R siRNA occurred at 24 h but peaked at 48 h and persisted 72 h after transfection. The effect of AT₁R siRNA on HEK 293 cells expressing AT_{1a} receptors was shown at 48 h after

transfection with AT₁R siRNA. $P < 0.01$ vs. control HEK 293 cells (***) and vs. HEK 293 cells expressing AT_{1a} receptors at 48 h after transfection (++).

Fig. 3.

Effects of AT₁R siRNA on AT₁ receptor expression in immortalized rabbit PTCs. The cells were transfected with an AT₁R siRNA or a scrambled siRNA for 48 h at 37°C. Nontransfected cells were used as a control. The cells were either stained with FITC-labeled ANG II for live cell imaging of receptor-mediated endocytosis of ANG II or lysed to extract proteins for Western blot of AT₁ receptor proteins (see MATERIALS AND METHODS for details and Refs. 33,34,54,56). *A*: fluorescence imaging of AT₁ receptor-mediated endocytosis of ANG II in nontransfected PTCs. *B*: the effect of losartan, which blocked FITC-ANG II labeling. *C*: fluorescence imaging of AT₁ receptor-mediated endocytosis of ANG II in AT₁R siRNA-transfected PTCs. *D*: Western blots of AT₁ receptor proteins in control, Val⁵-ANG II-treated, AT₁R siRNA-transfected, or scrambled siRNA-transfected cells. *E*: semiquantitative results of AT₁ receptor protein abundance. AT₁R siRNA knocked down AT₁ receptor proteins > 70%, whereas the scrambled siRNA had little effect. **P* < 0.05 vs. control. ##*P* < 0.01 vs. ANG II-treated PTCs.

Fig. 4.

Effects of AT₁R siRNA transfection, scrambled RNA, or losartan on AT₁ receptor-mediated accumulation of extracellular ANG II in immortalized rabbit PTCs. The cells were maintained serum free for 24 h before being treated without (control) or with Val⁵-ANG II (1 nM), AT₁R siRNA plus Val⁵-ANG II, scrambled RNA plus Val⁵-ANG II, or losartan (10 μM) plus Val⁵-ANG II for 60 min at 37°C. The cells were washed with 5 mM ice-cold acetic acid buffer containing 150 mM NaCl, pH 2.5, to remove cell surface-bound ANG II (37). ANG II was extracted from PTCs and assayed with a sensitive ANG II ELISA kit (Biochem) as described previously (34,56). *P* < 0.05 vs. control (*) and vs. Val⁵-ANG II treated (#); *n* = 6–12.

Fig. 5.

Effects of cold, colchicine, and phenylarsine oxide (PAO) on AT₁ receptor-mediated accumulation of extracellular ANG II in immortalized rabbit PTCs. The extent of AT₁R siRNA-inhibited ANG II endocytosis was comparable to that induced by cold, colchicine, or PAO, suggesting that the endocytotic machinery such as cytoskeleton microtubules and tyrosine phosphatases plays an important role in AT₁ receptor-mediated ANG II endocytosis in PTCs (34). * $P < 0.05$ or ** $P < 0.01$ vs. control. ## $P < 0.01$ vs. Val⁵-ANG II treated; $n = 6-12$.

Fig. 6.

Effects of AT₁ receptor knockdown by AT₁R siRNA on cell lysate NHE-3 protein abundance as determined by Western blot in immortalized rabbit PTCs. ANG II significantly increased NHE-3 protein abundance, whereas AT₁R siRNA blocked ANG II-induced responses. The effect was specific to AT₁ receptors because scrambled siRNA had no effect on ANG II-induced NHE-3 expression. $P < 0.05$ vs. control (*) and vs. Val⁵-ANG II treated (#); $n = 6$.

Fig. 7.

Effects of blockade of receptor-mediated ANG II endocytosis by cold or losartan on ANG II-induced NHE-3 expression in immortalized rabbit PTCs. Cold is known to stop receptor-mediated endocytosis, whereas losartan inhibits cell-surface AT₁ receptors. Both approaches significantly attenuated ANG II-induced increases in NHE-3 protein abundance, thus complementing the effects of AT₁R siRNA, which inhibits AT₁ receptor expression. $P < 0.05$ vs. control (*) and vs. Val⁵-ANG II treated (#); $n = 6$.

Fig. 8.

Effects of the endocytotic inhibitors colchicine and PAO on ANG II-increased NHE-3 protein abundance in immortalized rabbit PTCs. Both colchicine, which inhibits receptor endocytosis by disrupting cytoskeleton microtubules, and PAO, which blocks AT_1 receptor endocytosis by inhibiting tyrosine phosphatases, significantly attenuated ANG II-increased NHE-3 proteins. These findings suggest that AT_1 receptor-mediated endocytosis of extracellular ANG II may play a regulatory role in proximal tubule sodium transport by increasing NHE-3 expression. $P < 0.05$ vs. control (*) and vs. Val⁵-ANG II treated (#); $n = 6$.

Fig. 9.

Effects of AT₁R siRNA, losartan, and colchicine on ANG II-induced increases in apical biotinylated NHE-3 protein abundance in immortalized rabbit PTCs. To determine whether the effects of AT₁R siRNA, losartan, and colchicine on ANG II-induced cell lysate NHE-3 protein abundance were because of inhibition of NHE-3 synthesis or insertion in the cell membrane, PTCs were biotinylated before Western blot using apical membrane proteins. AT₁R siRNA, losartan, and colchicine all inhibited ANG II-increased biotinylated NHE-3 abundance, suggesting that both NHE-3 synthesis and insertion were affected by these treatments. $P < 0.01$ vs. control (**) and vs. Val⁵-ANG II treated (++); $n = 6$.

Fig. 10.

Effects of blocking AT₁ receptor-mediated ANG II endocytosis with AT₁R siRNA, losartan, and colchicine on ANG II-induced mitogen-activated protein (MAP) kinase extracellular signal-regulated kinase (ERK) 1/2 activation in immortalized rabbit PTCs. ANG II (1 nM) significantly increased MAP kinase ERK1/2 phosphorylation, whereas inhibition of AT₁ receptor-mediated ANG II endocytosis with AT₁R siRNA, losartan, or colchicine attenuated ANG II-induced MAP kinase ERK1/2 activation. $P < 0.01$ vs. control (**) and vs. Val⁵-ANG II treated (++); $n = 3$.